metabolism in certain tissues. Cross & Silver (1962) reported that the administration of Nembutal to rams did not change tissue oxygen concentrations in the testis and epididymis, and this anaesthetic was reported to be without effect on hepatic blood flow in dogs (Gilmore, 1958). Diminished acetate entry was probably due to impaired absorption from the rumen, since inhibition of rumen movements caused by anaesthesia would prevent adequate mixing of rumen contents. Acetate oxidation in rats was reported to be unaffected by Nembutal (Williams & Van Bruggen, 1956).

SUMMARY

1. A general procedure for studying the oxidative metabolism of substrates by specific tissues is described with particular reference to the metabolism of glucose and acetate by the testis and epididymis of the anaesthetized ram.

2. Continuous infusion of $NaH^{14}CO_3$ demonstrated the presence of bicarbonate pools in testicular and epididymal tissue. Equilibrium conditions between bicarbonate pools in the whole sheep were reached within about 300 min. after the start of infusion.

3. Glucose is a more important metabolite than acetate in testicular and epididymal metabolism, values for glucose and acetate uptake from blood based on arteriovenous differences being 5·0 and 3·0 μmoles/min./100 g. of tissue respectively.

4. The contributions of glucose and acetate to the overall oxidative metabolism of the testis and epididymis calculated by comparing the specific radioactivities of CO_2 produced by the tissue with those of the substrates in the circulation were 30 and 20% respectively. About one-third of the glucose and all of the acetate taken up from blood by the testis and epididymis was directly oxidized.

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The Effect of Ethanol and Electrical Stimulation on the Amino Acid Metabolism of Rat-Brain-Cortex Slices *in vitro*

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The object of the present study was to reproduce *in vitro* the changes in the concentrations of amino acids which had been observed in the brains of living rats during intoxication by ethanol (Häkkinen & Kulonen, 1961): a temporary increase in the concentrations of γ-aminobutyric acid, glutamic acid and aspartic acid and a decrease in the concentration of glutamine.

The metabolic relationships of glutamic acid and γ-aminobutyric acid have attracted great interest

(for general references see Roberts, 1960). Some of the artificially produced changes in the γ-aminobutyric acid concentration in the brain can be explained in enzymological terms. The administration of hydrazides was followed by depletion of γ-aminobutyric acid and by convulsions, which were prevented by direct application of the coenzyme of glutamate decarboxylase, vitamin B_6, or by the application of γ-aminobutyric acid itself (Killam & Bain, 1957; Killam, Dasgupta & Killam, 1960). A similar depletion of γ-aminobutyric acid was caused by 4-methoxymethylpyridoxine (Purpura, Berl, Gonzalez-Monteagudo & Wyatt, 1960) and by potassium cyanide poisoning (Tursky, 1960). Elevation of the γ-aminobutyric acid concentration in brain was achieved by the administration of hydroxylamine, which chiefly inhibits the γ-aminobutyrate transaminase (Baxter & Roberts, 1960; Eidelberg, Baxter, Roberts & Saldias, 1960). γ-Aminobutyric acid may act as a substrate for oxidative metabolism (McKhann & Tower, 1959).

In the present study the reproduction of the effects of ethanol *in vivo* was found possible with electrically stimulated slices only. The other main object of the study was to elucidate the mechanism of the observed changes in the concentrations of γ-aminobutyric acid and glutamine. Utilization experiments were therefore performed and the results confirmed by a study of the metabolism of ^{14}C-labelled glutamic acid. The results are consistent with the hypothesis that the catabolism of γ-aminobutyric acid is inhibited by ethanol. Part of the results has been presented as a preliminary communication (Kulonen, Häkkinen & Wallgren, 1960).

EXPERIMENTAL

Preparation and incubation of slices

All experiments were performed with conventional Warburg constant-volume respiratory manometers. The stimulators and electrode flasks were as described by Wallgren & Kulonen (1960), except for a slight change in the stimulators (Wallgren, 1961) that gave a pulse frequency of 100/sec. instead of the previous 180–200/sec. A potential gradient of 1·5–1·6 v/mm., as measured with an electronic voltmeter, was maintained between the electrodes.

Phosphate-buffered medium (McIlwain, 1951) containing glucose (6 mM) was used in all the experiments, the volume per flask being 2·5 ml. The gas phase was pure oxygen and the temperature 37·5°. Respiration was measured at 10 min. intervals during 60 min. unless otherwise stated.

Adult rats of Wistar origin were used. The preparation of the slices and the general procedure were as described by Wallgren & Kulonen (1960) except that four slices were cut from each hemisphere. Although white matter could be seen in the fourth slice, respiration was not lower in these than in previous experiments. The thickness of the slices seemed to be of considerable importance. This appeared in the experiments with radioactive glutamic acid, where in the third experimental series the slices were 20–25% heavier than in the first and second series. This difference caused some fluctuation in the results (Tables 5 and 6).

Usually, each experiment was set up with two electrode flasks and two ordinary flasks. Tissue was taken from one rat for stimulation and from the other for use without stimulation. Two slices from each cerebral hemisphere were placed in control medium and the other two were exposed to ethanol, each flask thus containing four slices weighing altogether 80–120 mg. When the flasks had been gassed, the ethanol (in 0·2 ml. of the experimental medium) was added to the side arms, and tipped into the main compartments after about 10 min. in the bath. The concentration of ethanol in the flasks was 0·4% (w/w) unless otherwise stated.

Amino acids were added to the incubation medium in about the same concentration in which they are present in brain tissue (Häkkinen & Kulonen, 1961). The additions were: γ-aminobutyric acid, 12·5 μmoles, or 1·29 mg./2·5 ml. of incubation solution; glutamic acid, 24 μmoles, or 3·53 mg./2·5 ml.; glutamine, 10 μmoles, or 1·46 mg./2·5 ml. The ammonia added was 0·3 mg./100 ml. or 23·5 μg. of NH_4Cl/2·5 ml. of incubation solution (Richter & Dawson, 1948). In the experiments with ^{14}C-labelled glutamic acid 21 μg. (0·5 μc) of glutamic acid was added as ammonium glutamate/2·5 ml. of incubation solution.

The amount of the sample solution taken for each analysis represented from 2 to 20 mg. of fresh brain tissue, depending on the amount of amino acids present in the solution.

Reagents

The ethanol added to the incubation medium was manufactured and purified, for spectrometric research, by the State Alcohol Monopoly of Finland. The γ-aminobutyric acid was produced by Fluka A.-G., Buchs, Switzerland, the L(+)-glutamic acid by E. Merck A.-G., Darmstadt, Germany, and the L(+)-glutamine by F. Hoffmann–La Roche and Co. Ltd., Basel, Switzerland. The radioactive glutamic acid was uniformly labelled L-[^{14}C]glutamic acid (L-[U-^{14}C]glutamic acid) (batch 27) from The Radiochemical Centre, Amersham, Bucks. The cation-exchange resin was Dowex 50 (mesh 200–400), manufactured by the Dow Chemical Co., Midland, Mich., U.S.A. The rest of the chemicals used were all produced by E. Merck A.-G., Darmstadt, Germany, and they were of either the 'pro analysi' or 'for chromatography' grade.

Analysis of amino acids

After incubation the contents of each flask (the tissue slices and the incubation solution) were homogenized in ice-cold 75% (v/v) ethanol, the total volume of homogenate being about 20 ml. The homogenates were centrifuged as described by Häkkinen & Kulonen (1961). The supernatants were evaporated in an air stream at room temperature and the residues were dissolved in 2 ml. of water. For removal of the excess of salts the solution was passed through a column (2·5 cm. × 10 cm.) of cation-exchange resin Dowex 50 (H^+ form), washed with 25 ml. of water and eluted with about 200 ml. of aq. 1 N-ammonia. The eluate was evaporated, and the residue was dissolved in 1·5 ml. of water. From this solution the amino acids were determined chromatographically as described in detail by Häkkinen & Kulonen (1961).

Accuracy of the determinations. To illustrate the reproducibility of the determinations, the following standard deviations of duplicates from the respective mean of each pair were calculated: γ-aminobutyric acid, ±4·8% (n = 54); glutamic acid, ±1·9% (n = 43); glutamine, ±3·7% (n = 51); aspartic acid, ±4·7% (n = 52); alanine, ±5·0% (n = 53); glycine plus serine plus taurine (combined), ±4·8% (n = 10). The variation was on the average about the same as in the experiments without treatment with ion-exchange resin (Häkkinen & Kulonen, 1961).

Analysis of radioactive metabolites

In the experiments with [U-^{14}C]glutamic acid several additional substances were isolated and collected, and their radioactivities were measured.

Carbon dioxide. The CO_2 formed during incubation was absorbed in 0·1 ml. of 20% (w/v) potassium hydroxide solution placed in the centre well of the flask. Immediately after incubation the centre well was thoroughly washed out with about 4 ml. of water. From this aqueous solution the CO_2 was precipitated with 3 ml. of saturated barium hydroxide solution in a centrifuge tube. The tightly closed tubes were left to stand for about 24 hr. The barium carbonate formed was then centrifuged and washed first with 10% (w/v) potassium hydroxide solution and then with 75% (v/v) ethanol. The precipitate was transferred to a steel planchet after suspension in 75% (v/v) ethanol and dried in an air stream at room temperature. The weights of the barium carbonate samples were unreasonably high and thus indicated that some CO_2 of the air had been adsorbed since no special precautions had been taken during the handling of samples. Therefore only the total radioactivities of the CO_2 samples could be calculated.

Lipids. After the contents of the incubation flask had been homogenized in 75% (v/v) ethanol and the homogenate centrifuged, the sediment obtained was dried in a small glass bowl. The dry residue was extracted first with chloroform saturated with water, then with chloroform–methanol (1:1, v/v) at room temperature, and finally with chloroform–methanol (1:1, v/v) at boiling point. All extractions were performed three times with 2 ml. of solvent (Lovern, 1955). The combined lipid extracts were evaporated and dried on the steel planchet.

Proteins. The dry residue from the lipid extraction was re-extracted three times with 2 ml. of boiling water. The extract (presumably containing glycogen among other material) was evaporated and dried on the steel planchets. The radioactivity of this material was considerable, and in samples from stimulated slices 1·5–2 times as high as in those from non-stimulated slices. Since the chemical composition of these extracts was not analysed, the radioactivities are not presented in detail.

In view of these exhaustive extractions, the residue was considered to be protein. To facilitate the plating, the material was dissolved in 2 ml. of 6 N-HCl by gentle heating, and the solution was transferred to the steel planchets and dried.

Amino acids. The radioactivity of the individual amino acids formed from [U-^{14}C]glutamic acid was measured as follows. The amino acids were chromatographed two-dimensionally, stained with ninhydrin and eluted with water, and the colour of the eluate was measured as described by Häkkinen & Kulonen (1961). Samples (3 ml., corresponding to 6 mg. of fresh brain tissue) of the same eluates were evaporated to dryness on the steel planchets.

The staining of the spots of [U-^{14}C]glutamic acid with ninhydrin and the consequent cleavage of the molecule resulted in a disproportionately large decrease in the radioactivity. When an amount of glutamic acid (corresponding to a radioactivity of 0·025 μc) was applied to a spot and then eluted without staining, it yielded a radioactivity of 36 400 counts/min. When a spot of the same amount was first stained with ninhydrin and then eluted, it yielded a radioactivity of 8800 counts/min., i.e. only about 25% of the whole C_5 chain, instead of the 80% expected. No radioactivity remained in the eluted spots. Because the preparation was reportedly to be uniformly labelled, the treatment with ninhydrin was repeated in different conditions. The concentration of the ninhydrin solution, into which the papers were dipped, was found important. When 0·2 and 0·6% solutions of ninhydrin were applied, the remaining radioactivity after staining was 54 and 35% of the original respectively. As a rule, the colour was allowed to develop at room temperature, and the heating of the papers at about 80° for 24 hr. did not affect the result. In all the experiments reported in the present paper, Whatman no. 1 chromatography paper was used, but with Schleicher und Schüll paper no. 2043 (manufactured by Carl Schleicher und Schüll, Dassel/Kr. Einbeck, Germany) and 0·2% ninhydrin solution, 74% of the original radioactivity remained. To conclude, we think that the excess of ninhydrin causes additional, unexplained, reactions. Therefore, in spite of these results, we do not doubt the uniformity of the labelling of the [U-^{14}C]glutamic acid employed. Throughout all the experiments the conditions of the ninhydrin treatment were kept constant.

Measurement of radioactivity. The counting was carried out with a Tracerlab model SC–16 windowless flow counter. At least 2000 impulses were counted in each measurement. All the plates were of a sufficiently low weight to be measured at finite thickness. The corrections for self-absorption losses were derived from correction curves based on data given by Yankwich and Weigl (Calvin, Heidelberger, Reid Tolbert & Yankwich, 1949).

RESULTS

Amino acid concentrations of rat-brain-cortex slices incubated under various conditions

The concentration of amino acids in the contents of the incubation flasks after 1 hr. of incubation of slices in the presence or absence of 0·4% (w/w) ethanol is given in Table 1. The incubation itself raised the concentration of glutamine and that of the combined fraction of glycine plus serine plus taurine, as compared with the control concentrations observed in the experiments *in vivo*. From the sum of the final concentrations it seems that both electrical stimulation and the presence of ethanol decreased the concentrations of amino acids below the values obtained under control conditions. Since stimulated and non-stimulated slices differ in their states of activity, the effects of ethanol in each of them can be considered separately. In stimulated tissue, the presence of ethanol

Table 1. *Concentration of amino acids in the contents of the incubation flasks after incubation of rat-brain-cortex slices under various conditions*

Incubation was for 1 hr. 'Control' means normal conditions, 'stimulated' stimulation with electrical pulses, 'control + ethanol' the addition of ethanol (final concn. 0·4%, w/w), and 'stimulated + ethanol' stimulation with electrical pulses in the presence of 0·4% (w/w) ethanol. Experimental details are given in the text. The values are expressed as μmoles of amino acid/100 g. of fresh tissue and are the means of two independent experiments. The effect of ethanol is presented as the percentage change of the amino acid concentration ('control + ethanol' against 'control'; 'stimulated + ethanol' against 'stimulated'). The amino acid concentrations *in vivo* were taken from Häkkinen & Kulonen (1961).

Amino acid determined	Condition	Final concn.	Effect of ethanol (%)
γ-Aminobutyric acid (concn. *in vivo* 430)	Control	440	—
	Stimulated	390	—
	Control + ethanol	320	−27
	Stimulated + ethanol	530	+38
Glutamic acid (concn. *in vivo* 910)	Control	1000	—
	Stimulated	910	—
	Control + ethanol	860	−14
	Stimulated + ethanol	1040	+15
Glutamine (concn. *in vivo* 390)	Control	550	—
	Stimulated	470	—
	Control + ethanol	520	−4
	Stimulated + ethanol	450	−4
Aspartic acid (concn. *in vivo* 280)	Control	280	—
	Stimulated	180	—
	Control + ethanol	250	−11
	Stimulated + ethanol	230	+25
Alanine (concn. *in vivo* 260)	Control	290	—
	Stimulated	280	—
	Control + ethanol	290	0
	Stimulated + ethanol	240	−17
Glycine + serine + taurine* (concn. *in vivo* 780)	Control	1430	—
	Stimulated	1390	—
	Control + ethanol	1420	−2
	Stimulated + ethanol	1260	+11
Sum (concn. *in vivo* 3050)	Control	3990	—
	Stimulated	3620	—
	Control + ethanol	3660	−8
	Stimulated + ethanol	3750	+4

* The average molecular weight 102 was used in the calculations.

caused an increase in the concentrations of γ-aminobutyric acid, glutamic acid and aspartic acid, and a decrease in the concentration of glutamine. These results were comparable both qualitatively and quantitatively with those obtained in the experiments *in vivo* (Häkkinen & Kulonen, 1961). In the stimulated tissue, ethanol produced also a decrease in the concentrations of alanine and of glycine plus serine plus taurine (combined), whereas no effect of ethanol on these amino acids was observed in the experiments performed *in vivo*. The effect of ethanol on non-stimulated slices seems to be different from the changes observed in the intact brain.

The effect of ethanol concentration (with application of a standard stimulation and an incubation time of 1 hr.) was studied separately (Fig. 1). The form of the curves of the amino acid concentrations resembled the analogous curve for the uptake of oxygen by non-stimulated slices (Wallgren, 1961).

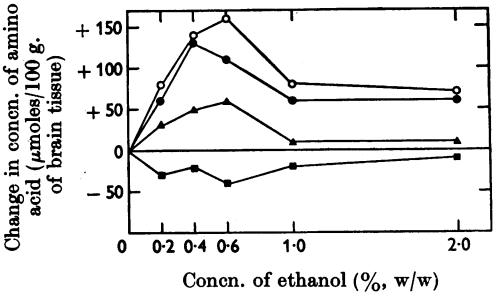

Fig. 1. Changes in the amino acid contents of rat-brain-cortex slices after the addition of various concentrations of ethanol to the incubation flasks. Standard stimulation and an incubation time of 1 hr. were applied, as described in the text. The changes are based on the values obtained without the addition of ethanol. Each point on the curves represents a mean of two independent experiments. ○, γ-Aminobutyric acid; ●, glutamic acid; ■, glutamine; ▲, aspartic acid.

The similarity may be fortuitous. Concentrations greater than 0·6 % (w/w) of ethanol have a generally depressing effect on amino acid metabolism. The changes in the concentrations of γ-aminobutyric acid and glutamic acid are of the same magnitude as was also observed in the experiments *in vivo*.

The effect of the duration of incubation is shown in Fig. 2. The form of the curves in Fig. 2 suggests that the primary change occurs in the metabolism of γ-aminobutyric acid. The concentrations of the amino acids did not persist at the changed level, although the concentration of ethanol presumably remained constant. The final decreasing trend of the elevated concentrations of the amino acids, glutamic acid, γ-aminobutyric acid and aspartic acid, is as yet unexplained. We believe it to result from the exhaustion of some metabolite that is not regenerated *in vitro*. Especially in our stimulated system the rate of respiration decreased during incubation.

Utilization of oxygen and amino acids by rat-brain-cortex slices

Table 2 shows the utilization of the amino acids, which were added in such amounts that their initial concentrations in the incubation solutions were the same as in fresh brain tissue. The concentrations of the amino acids inside the slices may be expected to differ to some extent from those in the incubation media. No attempt was made to study the slices and incubation fluid separately. The addition of γ-aminobutyric acid, glutamine and glutamic acid affected the oxygen consumption only slightly. In identical conditions, separate measurements of respiration alone were performed with the addition of γ-aminobutyric acid and glutamic acid (neutralized with sodium hydroxide; the sodium chloride concentration in the medium was decreased by the corresponding amount). The increase in the oxygen consumption of non-stimulated tissue was 15–20 % ($P < 0{\cdot}01$; $n = 10$) in the presence of each of these amino acids, and it was not significantly altered by the presence of 0·4 % (w/w) ethanol. In stimulated tissue, there was a small and statistically non-significant depression of respiration in the presence of γ-aminobutyric acid, whereas glutamic acid decreased oxygen uptake from 205 to 180 μmoles/g./hr. ($P < 0{\cdot}01$; $n = 11$). The presence of 0·4 % (w/w) ethanol did not modify this effect of glutamic acid. On the other hand, the respiratory depression caused by 0·9 % (w/w) ethanol in stimulated tissue was not altered by glutamic acid, a fact suggesting independent mechanisms of action as with ethanol and malonate (Wallgren, 1960).

The effect of stimulation on the utilization of glutamic acid in either the presence or absence of ethanol was small. Stimulation, as well as the presence of ethanol, increased the utilization of both γ-aminobutyric acid and glutamine. In stimulated slices ethanol diminished the utilization

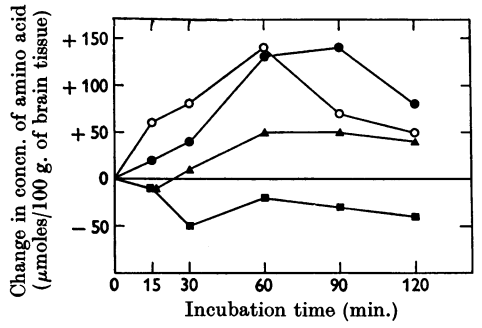

Fig. 2. Changes in the amino acid contents of rat-brain-cortex slices after incubation for various times. Ethanol (final concn. 0·4 %, w/w) was added to the incubation flasks and standard stimulation was applied, as described in the text. The changes are based on the values obtained without the addition of ethanol but with the incubation of the same duration. Each point on the curves represents a mean of two independent experiments. ○, γ-Aminobutyric acid; ●, glutamic acid; ■, glutamine; ▲, aspartic acid.

Table 2. *Utilization of added amino acids and oxygen by incubation of rat-brain-cortex slices under various conditions*

The conditions were as described in Table 1. The added amounts are listed in the text. The utilization of amino acids and oxygen is expressed in m-moles/100 g. of fresh tissue.

	Additions							
	None	γ-Aminobutyric acid		Glutamic acid		Glutamine		NH_4Cl
Conditions	O_2 consumed	Utilized	O_2 consumed	Utilized	O_2 consumed	Utilized	O_2 consumed	O_2 consumed
Control	13·4	4·17	14·6	15·6	12·9	5·00	14·2	13·4
Stimulated	19·5	6·98	18·5	14·5	16·9	6·99	19·7	19·5
Control + ethanol	13·0	5·72	15·3	16·7	14·6	5·69	14·4	13·6
Stimulated + ethanol	17·8	5·62	17·0	14·8	17·6	6·91	17·8	19·4

of γ-aminobutyric acid but did not affect the consumption of glutamine.

The conversion of the added amino acids into other amino acids in the slices is presented in Table 3. Only 21–29% of the utilized γ-aminobutyric acid and 9·8–16·6% of the glutamic acid were recovered as other amino acids. The fate of the residue is unknown. Since the addition of both glutamic acid and γ-aminobutyric acid increased the oxygen consumption of the non-stimulated slices, part of the added amino acid is presumably oxidized. Both stimulation and the presence of ethanol increase the net formation of γ-aminobutyric acid from glutamic acid and glutamine. If the rates of formation of other amino acids from γ-aminobutyric acid in stimulated slices in the presence and absence of ethanol are compared, it is seen that the conversion into glutamic acid, glutamine and aspartic acid is retarded by ethanol. This is also shown by the decreased utilization of γ-aminobutyric acid (Table 2). We can therefore tentatively conclude that ethanol prevents the catabolism of γ-aminobutyric acid. On the other hand, the addition of γ-aminobutyric acid stimulates the formation of glutamic acid and glutamine, as is expected, but this does not necessarily mean that the carbon chain is directly transformed. A relatively small part of the added glutamic acid is converted into γ-aminobutyric acid. When ammonium chloride was added, the concentration of glutamine increased at the expense of glutamic acid (Table 3).

In the compilation of Table 4, the concentration of the metabolically central glutamic acid has been taken as unity. The presence of ethanol caused a decrease in the concentration of glutamine and an increase in that of γ-aminobutyric acid in stimulated slices, even though the absolute values fluctuated with the different additions. This effect of ethanol on the net formation of γ-aminobutyric acid and glut-

Table 3. *Net conversion of added amino acids into other amino acids by incubation of rat-brain-cortex slices under various conditions*

The conditions were as described in Table 1, where the basic values also are given. The results give the changes in μmoles/100 g. of fresh tissue. The added amounts are described in the text and the utilizations are given in Table 2.

		Addition			
		γ-Aminobutyric acid	Glutamic acid	Glutamine	NH_4Cl
		Change in concn. of amino acid			
Amino acid determined	Condition				
γ-Aminobutyric acid	Control	—	+ 50	0	- 20
	Stimulated	—	+120	+ 80	- 10
	Control + ethanol	—	+120	+100	+ 60
	Stimulated + ethanol	—	+110	+ 60	- 30
Glutamic acid	Control	+300	—	+1210	-200
	Stimulated	+650	—	+1560	-160
	Control + ethanol	+570	—	+1420	-120
	Stimulated + ethanol	+430	—	+1480	-180
Glutamine	Control	+190	+510	—	+170
	Stimulated	+450	+760	—	+190
	Control + ethanol	+350	+660	—	+190
	Stimulated + ethanol	+320	+650	—	+170
Aspartic acid	Control	+ 70	+280	+200	+120
	Stimulated	+280	+570	+350	+120
	Control + ethanol	+150	+390	+270	+140
	Stimulated + ethanol	+200	+450	+330	+130
Alanine	Control	+ 60	+200	+ 60	+ 90
	Stimulated	+110	+240	+ 80	+ 90
	Control + ethanol	+100	+260	+100	+ 80
	Stimulated + ethanol	+140	+240	+150	+120
Glycine + serine + taurine*	Control	+250	+490	—	—
	Stimulated	+470	+720	—	—
	Control + ethanol	+420	+640	—	—
	Stimulated + ethanol	+540	+800	—	—
Sum	Control	+ 870 (21%)†	+1530 (9·8%†	—	—
	Stimulated	+1960 (28%)†	+2410 (16·6%)†	—	—
	Control + ethanol	+1590 (28%)†	+2070 (12·4%)†	—	—
	Stimulated + ethanol	+1630 (29%)†	+2250 (15·2%)†	—	—

* The average molecular weight 102 was used in the calculations.
† Of the total amount utilized.

amine could be confirmed by statistical evaluation of t, account being taken of the fact that the data are inherently paired (for γ-aminobutyric acid $P < 0.01$, for glutamine $P < 0.02$).

Metabolism of [U-^{14}C]glutamic acid in rat-brain-cortex slices

Table 5 shows the formation of CO_2, protein and lipid. By incubation in normal conditions a slightly larger proportion of glutamic acid was oxidized to CO_2 than in the other conditions. The amount of glutamic acid carbon incorporated into the total protein and total lipid was very small. Electrical stimulation diminished the incorporation into protein. In the present conditions the addition of ethanol did not influence the incorporation of the glutamic acid into protein. Quastel (1959) used higher concentrations of ethanol and found inhibition of the incorporation of glycine into the proteins of rat-brain-cortex slices. The concentration of lipid after 1 hr. of incubation was lower when ethanol had been present but the difference is not statistically significant.

Table 6 gives the results on the radioactive amino acids. The specific radioactivity of aspartic acid was close to that of glutamic acid in non-stimulated

Table 4. *Relative concentrations of γ-aminobutyric acid and glutamine after incubation of rat-brain-cortex slices with added amino acids*

The concentration of glutamic acid was taken to be 1·00. The values are based on Tables 1, 2 and 3. The conditions were as described in Table 1.

		Addition			
		None	γ-Aminobutyric acid	Glutamine	NH_4Cl
Amino acid determined	Condition	Relative concn. of amino acid			
γ-Aminobutyric acid	Control	0·44	—	0·20	0·53
	Stimulated	0·43	—	0·19	0·51
	Control + ethanol	0·37	—	0·18	0·51
	Stimulated + ethanol	0·51	—	0·23	0·58
Glutamine	Control	0·55	0·57	—	0·90
	Stimulated	0·52	0·59	—	0·88
	Control + ethanol	0·60	0·61	—	0·96
	Stimulated + ethanol	0·43	0·52	—	0·72

Table 5. *Conversion of [U-^{14}C]glutamic acid into carbon dioxide, total protein and total lipid by incubation with rat-brain-cortex slices under various conditions*

The conditions were as described in Table 1.

		CO_2		Protein		Lipid	
Condition	Expt. no.	10^3 × Radioactivity/μmole of O_2 consumed (counts/min.)	10^3 × Radioactivity/100 mg. of tissue (counts/min.)	Concn. of dry protein/fresh tissue (%, w/w)	Sp. radioactivity (counts/min./mg.)	Concn. of dry lipid/fresh tissue (%, w/w)	Sp. radioactivity (counts/min./mg.)
Control	1	—	—	13·0	38	2·8	93
	2	15·9	223	13·4	34	2·7	90
	3	12·5	160	13·0	37	0·8	143
	Mean	14·2	192	13·1	36	2·1	109
Stimulated	1	13·5	245	12·4	21	2·0	207
	2	9·6	190	14·2	23	1·6	183
	3	10·3	194	11·2	20	1·1	232
	Mean	11·1	210	12·6	21	1·6	207
Control + ethanol	1	—	—	12·7	36	1·0	165
	2	13·0	171	14·1	36	2·0	133
	3	10·7	142	11·0	32	0·8	165
	Mean	11·8	157	12·6	35	1·3	154
Stimulated + ethanol	1	13·1	225	12·9	27	1·7	221
	2	11·2	207	14·5	20	1·6	161
	3	9·0	156	12·8	18	0·7	221
	Mean	11·1	196	13·4	22	1·3	201

Table 6. *Conversion of* [U-^{14}C]*glutamic acid into some other amino acids by incubation with rat-brain-cortex slices under various conditions*

The conditions were as described in Table 1. Specific radioactivities are given in counts/min./μmole (after treatment with ninhydrin), concentrations in μmoles/100 g. of fresh tissue. The initial values of glutamic acid are calculated on the basis of dilution of the added labelled glutamic acid by the glutamic acid present in the slices.

		Glutamic acid				γ-Aminobutyric acid		Glutamine		Aspartic acid	
		Initial		Final		Final		Final		Final	
Condition	Expt. no.	Concn.	$10^3 \times$ Sp. radioactivity	Concn.	$10^3 \times$ Sp. radioactivity	Concn.	$10^3 \times$ Sp. radioactivity	Concn.	$10^3 \times$ Sp. radioactivity	Concn.	$10^3 \times$ Sp. radioactivity
Control	1	1050	190	1160	109	533	39·0	582	19·0	368	89·3
	2	1070	205	1190	130	485	35·0	554	18·5	398	119
	3	1030	158	1130	94	475	31·9	609	22·6	338	68·9
	Mean	1050	184	1160	111	498	35·3	582	20·0	368	92·4
Stimulated	1	1040	169	1090	121	456	22·8	452	11·0	301	34·0
	2	1040	170	1070	109	407	20·8	486	13·4	323	40·5
	3	1030	152	1070	94	475	28·8	534	17·4	323	28·8
	Mean	1040	164	1080	108	446	24·1	491	14·0	316	34·5
Control + ethanol	1	1050	183	1020	136	388	29·9	547	16·2	391	103
	2	1040	172	1050	136	359	30·6	513	16·6	376	96·8
	3	1020	147	1000	118	427	39·0	575	20·4	323	90·4
	Mean	1040	167	1020	130	391	33·2	545	17·8	363	96·7
Stimulated + ethanol	1	1060	189	1220	101	681	21·0	458	18·3	353	42·3
	2	1040	172	1180	94	592	19·8	445	17·1	338	37·8
	3	1020	137	1170	76	650	21·4	527	23·1	323	38·6
	Mean	1040	166	1190	90·5	621	20·7	477	19·5	338	39·6

Table 7. *Estimated formation and breakdown of γ-aminobutyric acid in rat-brain-cortex slices incubated with* [U-^{14}C]*glutamic acid under various conditions*

The conditions were as described in Table 1, and the calculations are given in the text. The results are taken from Table 6.

	Concn. of γ-aminobutyric acid (μmoles/100 g. of fresh tissue)			
	Formed during incubation	After incubation		Difference (breakdown)
Condition		Expected*	Observed	
Control	240	660	500	160
Stimulated	140	560	450	110
Control + ethanol	140	560	390	170
Stimulated + ethanol	190	610	620	−10

* By the sum of the pre-existing γ-aminobutyric acid (420 μmoles/100 g.) and the amount formed from the glutamic acid during the incubation period.

slices but on stimulation it decreased markedly, indicating an increased dilution by non-labelled metabolites. The decreased specific radioactivity of aspartic acid in stimulated slices may indicate difficulty in incorporation of the glutamic acid into the citric acid cycle. After incubation with electrical pulses the final relative specific radioactivity of the aspartic acid (that of glutamic acid being taken as 1·00) was 0·40–0·49 in the presence of ethanol, but only 0·28–0·37 in the absence of ethanol. The presence of ethanol did not affect the ratio of labelled CO_2 formed to the oxygen consumed.

To get a rough estimate of the kinetics of the γ-aminobutyric acid metabolism, the formation of γ-aminobutyric acid from glutamic acid was calculated on the basis of the formula:

$$ds_M/dt = (dn_{I\rightarrow M}/M)(s_I - s_M)$$

where I is the concentration of a single precursor (glutamic acid), M is the concentration of a product (γ-aminobutyric acid), s_I and s_M are the respective specific radioactivities and $dn_{I\rightarrow M}$ is the number of molecules of I transformed into M in time dt (Zilversmit, Entenman & Fishler, 1943). Since information is available for the first 1 hr. interval, which begins with s_M equals 0, ds_M/dt was first substituted by $\Delta s_M/\Delta t$, which gives the value s_M when Δt equals 1 hr. This was thought to be justified since the increase of γ-aminobutyric acid is approximately constant during the first hour (Fig. 2). The results are given in Table 7. They confirm the tentative conclusion, reached above, that the cause of the accumulation of γ-aminobutyric acid in the presence of ethanol and electrical stimulation is the inhibition of its breakdown. The renewal of γ-aminobutyric acid is rather slow. McKhann, Albers, Sokoloff, Mickelsen & Tower (1960) calculated the rate of metabolism of glutamic acid to γ-aminobutyric acid by using the same equation, but substituting for the specific radioactivities the estimated average specific radioactivities during the particular time-interval. If we used their formula for the calculation, the 'breakdown' of γ-aminobutyric acid in stimulated slices in the presence of ethanol would give even higher negative value than that given in Table 7.

The results on glutamine cannot be treated similarly, since its concentration does not decrease at a constant rate during the 1 hr. interval. However, the high total and specific radioactivities of glutamine indicate that an increased breakdown of glutamine occurs on stimulation in the presence of ethanol (Table 6).

DISCUSSION

One conclusion from this study is that only in the stimulated preparation was it possible to reproduce *in vitro* the effect induced by ethanol *in vivo*.

There is a tendency for glutamine to be depleted during ethanol intoxication, and added glutamine may act as a substitute and in this way counteract the intoxication. Ethanol intoxication in rats is alleviated by simultaneous oral administration of glutamine (Häkkinen & Kulonen, 1961; Sammalisto, 1962). On the possible changes in free ammonia no information is available, but the increase of glutamine on incubation is presumably a result of the liberation of ammonia from the amino acids. In slices prepared in the ordinary way and respiring in glucose-containing salt solutions, ammonia is liberated (McIlwain, 1959). Nothing is known about the effect of electrical stimulation on amino acid metabolism, but Kini & Quastel (1959) found that stimulation with K^+ ions increased the yield of glutamine and γ-aminobutyric acid from glucose.

The accumulation of γ-aminobutyric acid in the presence of ethanol is observed in stimulated slices only. Therefore it cannot be caused by a direct action of ethanol on the enzymes. We suggest that the combined effect of ethanol and stimulation causes a change in the metabolites or cofactors that regulate the catabolism of γ-aminobutyric acid. According to Elliott & Florey (1957), 'factor I' (γ-

aminobutyric acid) may be present in the brain in inactive form and therefore is metabolically inert. Our analytical procedure is likely to comprise also that γ-aminobutyric acid which may be occluded in subcellular particles or vesicles.

The low incorporation of ^{14}C from labelled glutamic acid into the lipid and protein fractions is in agreement with the results obtained by Davison (1961), who found that of intraperitoneally injected [1-^{14}C]glycine relatively little was incorporated into the proteolipid protein of the central nervous system of an adult rat.

In cat-brain-cortex slices, McKhann *et al.* (1960) have estimated the average rate of conversion of glutamic acid into γ-aminobutyric acid during 1 hr. of incubation to be 12·4 μmoles/g./hr. This is higher than the values calculated from Table 7. We believe that the disagreement is due to the different animal species and the different conditions of incubation, which are also evidenced in the rate of respiration. No certain conclusions can be drawn from experiments *in vitro* on the quantitative aspects of the γ-aminobutyric acid metabolism in a living animal.

In experiments with [U-^{14}C]glutamic acid (Table 5: conditions 'control', Expts. 2 and 3) the average total production of ^{14}C-labelled CO_2 was equivalent to 192 000 counts/min./100 mg. of tissue. The specific radioactivity of the glutamic acid in the beginning of the experiment was 785 000 counts/min./μmole (before treatment with ninhydrin; see the Experimental section). From this it would appear that 0·244 μmole of labelled glutamic acid had been metabolized to CO_2/100 mg. of tissue, which would need 1·10 μmoles of oxygen (4·5 μmoles of O_2/μmole of glutamic acid). However, if all the CO_2 had been evolved directly from the glutamic acid, the production of labelled CO_2/μmole of oxygen consumed would thus have been equivalent to 174 500 counts/min. (i.e. 192 000/1·10 or directly 785 000/4·5 counts/min.) instead of the 14 200 counts/min. observed (Table 5). This must mean that only about 8% of the total CO_2 produced originated from the glutamic acid. These calculations will not be exact if the ^{14}C was not uniformly distributed in the glutamic acid.

McKhann *et al.* (1960) estimated that the proportion of the total oxidation of the glutamic acid occurring via the γ-aminobutyric acid pathway was 43·8%. In our experiment, after the incubation period of 1 hr., the specific radioactivity and total radioactivity of γ-aminobutyric acid were 38% and 52% respectively of the respective values for aspartic acid (which comprises also the pathway through α-oxoglutarate). In the stimulated condition the capacity of the glucose–α-oxoglutaric acid–succinic acid pathway seems to be increased and the incorporation of glutamic acid into the citric acid cycle relatively to be decreased, as evidenced by the low radioactivities of the aspartic acid fraction (Table 6).

Cohen, Simon, Berry & Chain (1962) incubated guinea-pig-cerebral-cortex slices in media containing [^{14}C]glutamic acid. The results indicated 'aspartate formation from glutamate without major contribution by transamination to α-oxoglutarate or re-entry into the tricarboxylic acid cycle through conversion into succinate'. The relative specific radioactivity of aspartate was 0·92 ± 0·06 and that of γ-aminobutyrate 0·2 (the specific radioactivity of glutamate was taken to be 1·00). These findings agree well with the results on non-stimulated slices presented in Table 6. Also, with regard to the effect of stimulation, Professor J. H Quastel and his co-workers (personal communication) obtained, by stimulation with K^+ ions, a decrease in the specific radioactivity of aspartic acid that was formed by incubation in the presence of [^{14}C]glutamic acid.

SUMMARY

1. The changes observed *in vivo* in rat brain during ethanol intoxication could be reproduced when rat-brain-cortex slices were incubated in the presence of ethanol and simultaneously stimulated with electrical pulses: the concentrations of γ-aminobutyric acid, glutamic acid and aspartic acid increased above the control values, and the concentration of glutamine decreased. The effects of ethanol concentration and incubation time were recorded.

2. The concentrations of glutamine and of the combined serine plus glycine plus taurine fraction in rat-brain-cortex slices increased during incubation in normal conditions. In incubated non-stimulated slices ethanol caused only a general decrease in the concentrations of the amino acids. A similar effect was also obtained with stimulation in the absence of ethanol.

3. Both electrical stimulation and the presence of ethanol independently enhanced the utilization of γ-aminobutyric acid and glutamine, but not appreciably that of glutamic acid. The interconversion of γ-aminobutyric acid and glutamine into other amino acids was also enhanced, but only 10–30% of the amino acids utilized were recovered as other amino acids.

4. When the slices were stimulated in the presence of ethanol, the utilization of γ-aminobutyric acid (but not that of glutamine or that of glutamic acid) was smaller than in the absence of ethanol.

5. The incorporation of ^{14}C from labelled glutamic acid into lipids and proteins was very small in incubated rat-brain-cortex slices.

6. Stimulation markedly decreased the specific radioactivity of the aspartate formed in the presence of the ^{14}C-labelled glutamic acid.

7. The approximate rate of conversion of glutamic acid into γ-aminobutyric acid in rat-brain-cortex slices was estimated. It was concluded that the increase of γ-aminobutyric acid in the presence of ethanol *in vivo* and in stimulated slices is due to an inhibition of its breakdown. This effect is not direct, since it was not observed in non-stimulated slices.

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Purine and Pyrimidine Derivatives in Mature Pea Seeds

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In a recent investigation, the acid-soluble nucleotides of mature pea seeds were described and determined (Brown, 1962). However, except for a few reports describing the isolation of individual compounds (e.g. Charbuliez & Bernhard, 1932; Ritthausen, 1876, 1881; Fosse, deGraeve & Thomas, 1932), little is known of other purine and pyrimidine derivatives in seeds. To obtain a more complete picture, mature pea seeds have been examined with the main aim of identifying and determining the relative concentrations of such compounds. The analytical technique used was based on ion-exchange chromatography; anion-exchange rather than cation-exchange chromatography was employed in order to take advantage of the absence of glycosidic lability under alkaline conditions (Cohn, 1955).

MATERIALS AND METHODS

Preparation of seed extract. Mature seeds of *Pisum sativum* L. var. Meteor obtained from Suttons Seeds Ltd., Reading, and showing 88% germination, were used. Purines, pyrimidines, and their derivatives, were extracted